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Synergistic effect of folate-mediated targeting and verapamil-mediated P-gp inhibition with paclitaxel -polymer micelles to overcome multi-drug resistance

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ABSTRACT

Multidrug resistance (MDR) in tumor cells is a significant obstacle for successful cancer chemotherapy. Overexpression of drug efflux transporters such as P-glycoprotein (P-gp) is a key factor contributing to the development of tumor drug resistance. Verapamil (VRP), a P-gp inhibitor, has been reported to be able to reverse completely the resistance caused by P-gp. For optimal synergy, the drug and inhibitor combination may need to be temporally colocalized in the tumor cells. Herein, we investigated the effectiveness of simultaneous and targeted delivery of anticancer drug, paclitaxel (PTX), along with VRP, using DOMC-FA micelles to overcome tumor drug resistance. The floate-functionalized dual agent loaded micelles resulted in the similar cytotoxicity to PTX-loaded micelles/free VRP combination and co-administration of two single-agent loaded micelles, which was higher than that of PTX-loaded micelles. Enhanced therapeutic efficacy of dual agent micelles could be ascribe to increased accumulation of PTX in drug-resistant tumor cells. We suggest that the synergistic effect of folate receptor-mediated internalization and VRP-mediated overcoming MDR could be beneficial in treatment of MDR solid tumors by targeting delivery of micellar PTX into tumor cells. As a result, the difunctional micelle systems is a very promising approach to overcome tumor drug resistance.

1. Introduction

Paclitaxel (PTX), one of the most successful anticancer drugs, is the first of a new class of microtubule stabilizing agents, and has been demonstrated significant antitumor activity in clinical trials against a broad range of solid tumors, especially against non-small-cell lung cancer, metastatic breast cancer and refractory ovarian cancer [1,2]. However, because of low therapeutic index and the poor aqueous solubility of approximately 1 μg/ml of PTX, the only available commercial preparation of PTX is Taxol, a concentrated solution containing 6 mg PTX/ml of Cremophor EL (polyoxyl 35 castor oil) and dehydrated alcohol (1:1, v/v), which is diluted 5–20-fold in normal saline or dextrose solution before administration [1]. Unfortunately, serious side effects, such as hypersensitivity, neurotoxicity, nephrotoxicity, endothelial and vascular muscles causing vasodilatation, labored breathing and lethargy attributable to intravenous administration of the current Cremophor EL-based formulation have been reported [3]. For this reason the extensive clinical application of this drug is extremely limited [2].

Another major problem in the clinical treatment of cancer with PTX is multi-drug resistance (MDR) in tumor cells. MDR is a frequent phenomenon whereby cancer cells become resistant to the cytotoxic effects of various structurally and mechanistically unrelated chemotherapeutic agents [4]. Once the MDR occurred, the intracellular drug accumulation reduced and the sensitivity of tumor cells to drugs was significantly decreased. Therefore, development of MDR is a major obstacle to the success of cancer chemotherapy. In past years, various mechanisms of MDR have been proposed, including the over-expression of multi-drug efflux pumps like P-glycoprotein (P-gp) [5], reduces in topoisomerase activity [6], modifications in glutathione metabolism [7], and altered expression of apoptosis-associated protein Bcl-2 [8] and tumor suppressor protein p53 [9]. Of these mechanisms, overexpression of P-gp encoded by the MDR1 is the most commonly encountered in successful cancer therapy with PTX [10]. P-gp, a drug efflux transporter, which can result in an increased efflux of the cytotoxic drugs from the cancer cells, thus lowering their intracellular concentrations. In many cancer types, nearly 40–50% of the patients diagnosed with cancer have P-gp up to 100-fold over-expression in the malignant tissue [11,12].

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An attempt to overcome P-gp-based MDR in cancer chemotherapy is to co-administer a P-gp inhibitor along with the anticancer drug [13–16]. Verapamil (VRP), a first generation P-gp inhibitor, has been reported to be able to reverse completely the resistance caused by P-gp *in vitro* at concentrations of approximately 5–10 μM [17]. However, cardiovascular toxicity was observed when VRP plasma concentration reached 1–2 μM *in vivo* [18]. Meanwhile, the differences in physico-chemical properties of the anticancer drug and P-gp inhibitor may result in differences in the pharmacokinetics and tumor accumulation of the two agents. Both the drug and the inhibitor may need to be simultaneous located in the tumor cells for optimal synergy. That is to say, the two agents should minimal exposure to the normal tissues and temporal colocalization in tumors.

Due to these problems, there is a need for the development of alternate formulation of PTX having good aqueous solubility, reducing side effects and at the same time overcoming MDR in tumor cells. Accordingly, a number of alternative formulations were investigated for solubilization of PTX, including nanoparticles, liposomes, microspheres, PTX-polymer conjugates, dendritic polymers, implants, water-soluble prodrugs etc [2,19]. Although these vehicles employed have shown a lot of promise to replace the Cremophor EL-based vehicle for PTX delivery, approaches overcoming the MDR of tumor cells have seldom been considered. Therefore, to overcome the MDR of tumor cells, decrease the side effects and improve the effectiveness of PTX and VRP, it is a potential approach to co-encapsulating the two agents by using a targeted delivery system.

In our previous work, O-carboxymethylated chitosan (OCMC) was firstly hydrophobically modified with deoxycholic acid (DOCA), then covalently bound with folic acid (FA) to develop a new cancer-targeted drug delivery system (DOMC-FA) [20]. In this paper, DOMC-FA micelles were used as a biodegradable drug carrier for simultaneous encapsulation of PTX and VRP. To evaluate whether folate-functionalized dual agent micelles (DOMC-FA/(PTX + VRP) formulation) possesses ability in overcoming tumor drug resistance, MTT assay, morphological study, cell cycle analyz and Annexin V-FITC/PI staining were investigated in MCF-7/ADR cells.

2. Materials and methods

2.1. Materials

DOMC and DOMC-FA conjugates were prepared in our lab described previously [20]. 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were obtained from Sigma Co., Ltd. (USA). Paclitaxel (PTX) was obtained from Xian Haoxuan Bio-tech Co., Ltd. (Xi an, China). Verapamil hydrochloride (VRP·HCL) was purchased from Aladdin chemistry Co., Ltd. (Shanghai, China). Taxol injection (Anzatax Injection Concentrate,30 mg/5 ml) was produced by Beijing Shiqiao Biological Pharmaceutical Co., Ltd. (Beijing, China). Penicillin–streptomycin, RPMI-1640 medium, folate-free RPMI-1640 medium (R1145), fetal bovine serum (FBS), 0.25% (w/v) trypsin–0.03% (w/v) EDTA solution and Phosphate buffer solution (PBS) were purchased from Gibco BRL (Gaithersberg, MD, USA). Propidium iodide (PI), hoechst 33342, RNase A and Annexin V-FITC were purchased from KeyGen bio-technology (Nanjing, China). Breast cancer cell lines MCF-7 and MCF-7/ADR were kindly donated by the Department of Pharmacology, School of Pharmacy, Shandong University. All reagents were analytical grades and used without further purification. Water was purified by distillation, deionization, and reverse osmosis (Milli-Q plus).

2.2. Preparation of PTX or/and VRP-loaded micelles

The durg-loaded micelles were prepared by a self-assemble method as detailed in our previous report [20]. In brief, 25 mg of DOMC or DOMC-FA conjugates was mixed with 15 mg of PTX or VRP (for the co-encapsulated micelle the added quality of PTX and VRP was 170.8 μg and 22.73 mg respectively) in 5 mL distilled water, followed by gentle shaking at room temperature for 3 h. Then the solution was sonicated three times using a probe-type sonifier (JY92-Ultrasonic Processor Xinzhi, Linbo,China) at 90 W for 2 min each under the ice bath condition. The pulse was turned off for 2 s with the interval of 4 s to inhibit increase in temperature. Then, the mixture was centrifuged at 5000 r/min for 20 min to remove the unloaded monomer and passed through membrane filter (pore size: 0.45 μm, Millipore), followed by lyophilization or stored at 4 °C for use. The content of PTX and VRP were determined by HPLC assay after the disruption of the micelles and the solubilization of PTX and VRP in acetonitrile. Before loading VRP to the micelles, verapamil hydrochloride (VRP·HCL) was stirred with 1.5 equiv. mole of NaOH (0.1 M) to obtain the VRP base.

2.3. Characterization of micelles

2.3.1. Determination of drug-loading parameters

PTX was extracted from the polymer micelles with acetonitrile, then filtered with a 0.2 mm syringe filter and analyzed for its concentration using high-performance liquid chromatography (HPLC, Agilent 1100 series , USA). Sample solution was injected at least three times at a volume of 20 μl into a Phenomenex-ODS C18 column (150 × 4.60 mm, 5 μm) preceded by a C18 guard column (Dikma, China). The mobile phase used was a mixture of water and acetonitrile in the volume ratio of 53:47. The elution rate was 1.0 mL/min and the paclitaxel detection wavelength was set at 229 nm.

Compared to that of incorporated PTX as shown later (>2.5 mg/mL), the solubility of free PTX in water is very low (<1 μg/ml). So the amount of free PTX in this preparation was not taken into account [21].

The content of VRP in micelles was separated from the aqueous micelles suspension by ultrafiltration (AmiconR Ultra-15, MWCO = 100,000) at 4000 rpm for 20 min and measured by HPLC. The mobile phase, consisting of acetic acid-acetic natrium: methanol: three-ethylamine (45:55:1), was delivered at a flow rate of 1.0 mL/min. Eluted compounds were detected at 278 nm using a UV–Vis detector.

The drug concentration of PTX and VRP were calculated from standard curves. The assay was linear over the tested concentration range, and there was no interference of the polymer with the assay. Drug-loading efficiency (DL) and encapsulation efficiency (EE) were calculated as following:

$$\mathrm{DL\%} = \frac{\text{weight of the drug in micelles}}{\text{weight of the feeding polymer and drug}} \times 100\%$$

$$\mathrm{EE\%} = \frac{\text{weight of the drug in micelles}}{\text{weight of the feeding drug}} \times 100\%$$

2.3.2. Evaluation of particle size and zeta potential

The average particle size and size distribution of drug-loaded micelles were determined using a Zetasizer (3000HS, Malvern Instruments Ltd, UK). The polydispersity index range was comprised between 0 and 1. Zeta potentials of micelles were measured with a zeta potential analyzer (Brookhaven ZetaPALS, USA). The concentration of micelles was kept constant at 2.0 mg/mL. Each sample was determined triplicate.

2.3.3. Transmission electron microscopy (TEM) observation

The morphology and size of micelles were observed using a transmission electron microscope (TEM) (H-7000, Hitachi, Japan). A drop of sample solution (2 mg/mL) was placed onto a 300-mesh copper grid coated with carbon. After 2 min, the grid was tapped with a filter paper to remove surface water, followed by air drying and negatively stained with 2% phosphotungstic acid for 30 s. The grid was dried at room temperature and then observed by TEM.

2.3.4. X-ray diffraction

X-ray diffraction (XRD) spectrometry was obtained using an XD-3A powder diffractometer (D/Max r-B, Rigaku, Japan). A Cu Kα radiation at 40 kV and 100 mA was used. Diffractograms were performed from the initial angle $2\theta = 3^\circ$ to the final angle $2\theta = 50^\circ$ with the steps of 0.02°, at a scanning speed of 4°/min (2θ).

2.4. In vitro drug release studies

In order to create pseudo-sink conditions, the *in vitro* drug release from micelles was determined in phosphate buffer saline (PBS, 0.15 M, pH 7.4 and 6.0) containing 0.5% w/v Tween 80. One milligram of drug-loaded micelles was filled into a dialysis tube (molecular weight cutoff = 12,000, Spectrum®), and the endsealed dialysis tube was immersed fully in 150 mL of the release medium in a erlenmeyer flask. The erlenmeyer flasks were placed in an incubator at 37 ± 0.5 °C with stirring at 100 rpm. At predetermined time intervals, 1 ml of the release medium was withdrawn and replaced with an equal volume of fresh release medium. The collected samples were lyophilized and then dissolved in 1 mL of mobile phase and analyzed by HPLC under the same analytic conditions as described above. The results of triplicate measurements were used to calculate cumulative drug release.

2.5. In vitro cell culture studies

2.5.1. Cell line and cell culture

MCF-7/ADR (multi-drug-resistant variant), a human breast carcinoma cell line that has been induced by doxorubicin, and MCF-7 (human breast cancer cells), the parental line were kindly donated from Department of Pharmacology, School of